Mast Cells Induce Vascular Smooth Muscle Cell Apoptosis via a Toll-Like Receptor 4 Activation Pathway

Wijnand K. den Dekker, Dennie Tempel, Ilze Bot, Erik A. Biessen, Leo A. Joosten, Mihai G. Netea, Jos W. M. van der Meer, Caroline Cheng, Henricus J. Duckers

Objective—Activated mast cells (MCs) release chymase, which can induce vascular smooth muscle cell (VSMC) apoptosis leading to plaque destabilization. Because the mechanism through which MCs release chymase in atherosclerosis is unknown, we studied whether MC-associated VSMC apoptosis is regulated by toll-like receptor 4 (TLR4) signaling.

Methods and Results—Local recruitment and activation of MCs reduced VSMC content specifically in the cap region of vulnerable plaques in apolipoprotein E knockout mice. Cotreatment with the TLR4 antagonist Bartonella quintana lipopolysaccharide prevented this VSMC loss, suggesting an important role for TLR4 signaling in MC-induced VSMC apoptosis. Coculture of VSMCs with MCs activated by the TLR4 agonist Escherichia coli lipopolysaccharide increased VSMC apoptosis. Apoptosis was inhibited by TLR4 and chymase blockers, indicating that TLR4 signaling is involved in chymase release in MCs. This pathway was mediated via interleukin-6 because interleukin-6 promoted MC-associated VSMC apoptosis, which was inhibited by blocking chymase release. In addition, TLR4 activation in MCs induced interleukin-6 production, which was reduced by preincubation with either B. quintana lipopolysaccharide or an anti-TLR4 antibody.

Conclusion—We show that MCs promote VSMC apoptosis in vivo. In addition, TLR4 signaling is important in chymase release in MCs and, therefore, in plaque destabilization by regulating VSMC apoptosis. (Arterioscler Thromb Vasc Biol. 2012;32:0-0.)

Key Words: apoptosis ■ mast cell ■ toll-like receptor 4 ■ vascular smooth muscle cell ■ vulnerable plaque

Atherosclerosis is regarded as an inflammatory-driven disease in which macrophages and T lymphocytes are well-known players and abundantly present in the diseased vascular wall.1–3 Macrophages ingesting oxidized lipids, thus forming foam cells, are one of the first steps in forming a fatty streak, the earliest form of atherosclerosis. A complex interplay among inflammatory cells, cytokines, and degrading enzymes may lead to progression of an advanced atherosclerotic plaque into a rupture-prone unstable plaque. Rupture of this vulnerable plaque, characterized by a lipid-rich necrotic core, high macrophage, low vascular smooth muscle cell (VSMC) content, and a thin fibrous cap (<65 μm),4 is considered to be the major cause of an acute myocardial infarction.5 Besides macrophages and T lymphocytes, there is accumulating evidence that mast cells (MCs) are also important mediators in determining the phenotype of an atherosclerotic plaque.6,7 Activated MCs may contribute to vulnerable plaque formation via different mechanisms. Bot et al.8 showed that MCs can induce plaque destabilization in a murine model for atherosclerosis, associated with an increase in intraplaque hemorrhaging because of enhanced vascular permeability and enlargement of the lipid-rich necrotic core because of macrophage apoptosis. Furthermore, MCs can produce a wide pallet of proinflammatory cytokines (eg, interleukin-6 [IL-6],9 can induce VSMC apoptosis,10 and activate matrix metalloproteinases.11–13 All these processes might lead to weakening of the fibrous cap and subsequently a higher risk of plaque rupture. Indeed, it has been shown that activated MCs are mainly located in the shoulder region of coronary plaques, which is the region with the highest chance of rupture.14 Increased risk of plaque rupture in vulnerable plaque is associated with VSMC apoptosis,10,15,16 which weakens the VSMC-rich fibrous cap.17 In vitro and in vivo, MCs are capable of inducing VSMC apoptosis by chymase release.10,18 The effect of chymase release and the mechanism through which MCs are activated to release chymase in the vulnerable plaque remain to be elucidated. Toll-like receptor 4 (TLR4) is an important pattern recognition receptor of the innate immune system.19 Activation of TLR4 activates a downstream signaling pathway that induces nuclear translocation of nuclear factor κB and subsequent transcription of proinflammatory cytokines, including IL-6.20 It has previously been shown.

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that MCs express TLR4, and more recently, Avila and Gonzalez-Espinosa\textsuperscript{21} reviewed the importance of TLR4 on MCs in the immune reaction against gram-negative bacteria. Because it has been shown that IL-6 can upregulate chymase protein expression in human MCs,\textsuperscript{22} we hypothesized that TLR4 signaling is involved in chymase release in MCs. We investigated the role of TLR4 signaling in MC activation and described the subsequent effect on MC-induced VSMC apoptosis, both in a validated murine vulnerable plaque model and in vitro using coculture experiments.

**Materials and Methods**

**Animal Model**

All animal work was performed in compliance with the guidelines issued by the Dutch government and was approved by our local animal welfare committee. We used 12-week-old female apolipoprotein E knockout (C57BL/6) mice (Jackson Laboratory, Cambridge, United Kingdom). Vulnerable plaque formation was induced as described earlier.\textsuperscript{17,24} In short, after 2 weeks of acclimatization, mice were fed a Western diet (containing 15\% [wt/wt] cacao and 0.25\% [wt/wt] cholesterol; Arie Blok, Utrecht, the Netherlands). Two weeks after initiation of Western diet, a tapered flow-altering cast was surgically implanted around the right common carotid artery. Eight and 9 weeks after cast placement, Pluronic F-127 gel (Sigma-Aldrich, Zwijndrecht, the Netherlands), containing different pharmacological compounds or PBS placebo, was applied periventricularly, upstream of the cast. These compounds included the natural TLR4 antagonist \textit{B. quintana} lipopolysaccharide (LPS), isolated as previously described,\textsuperscript{25} the functional grade purified TLR4 antibody (TLR4a; eBioscience, San Diego, CA) or 2,4-dinitrophenyl-human serum albumin (DNP-HSA; Sigma-Aldrich), which attracts and activates specifically MCs.\textsuperscript{5} The mice were randomly assigned to the treatment groups (n=8 per group; Table). Three days after the second application of Pluronic F-127 gel, mice were euthanized, and the carotid artery was dissected and embedded in optimal cutting temperature compound (Sakura Finetek, Alphen aan den Rijn, the Netherlands), snap-frozen in liquid nitrogen, and stored at –80°C until further processing. The findings of this first experiment were further validated in 2 additional in vivo studies. In the first study, 1 group of mice (n=6) was treated with lentivirus coding for a short hairpin RNA sequence that targets murine TLR4 to achieve local silencing of the gene whereas the second group of mice (n=6) was treated with sham lentivirus. Both lentiviruses were applied around the carotid artery 8 weeks after cast placement. One week later, we applied Pluronic F-127 gel containing DNP-HSA around the carotid artery in all animals. Three days after the application of the gel, we euthanized the animals and took out the carotid artery. In the second study, we used cromolyn as an alternative strategy to stabilize the MCs. Two groups of mice (n=4 per group) received an intravenous injection of cromolyn (25 mg/kg) or PBS, 30 minutes before local DNP-HSA challenge. During the challenge, the mice received twice daily an intraperitoneal injection of cromolyn (50 mg/kg) or PBS. Three days after the start of the challenge, mice were euthanized, and the carotid arteries were again taken out and processed.

**Histology and Immunohistochemistry**

The carotid artery region upstream of the cast was serially sectioned in 6-μm sections. Histological and immunohistochemical analysis was performed in 72-μm intervals, covering the whole vulnerable plaque. MCs were stained by toluidine blue (Sigma-Aldrich). Nonactivated and activated MCs were distinguished by bright-field assessment of cell morphology after toluidine blue staining. Activated MCs show degranulation and release of toluidine blue–positive remnants whereas nonactivated MCs show intracellular and intravesicular accumulation of toluidine blue. In addition to toluidine blue staining and routine hematoxylin-eosin staining, different immunohistochemical stainings were performed to evaluate plaque stability. Sections were stained for VSMCs (anti–α-actin; Sigma-Aldrich), apoptotic cells (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] assay; Roche, Woerden, the Netherlands), macrophages (anti-CD68 antibody; AbD Serotec, Raleigh, NC), endothelial cells (anti–platelet endothelial cell adhesion molecule 1 antibody; BD Biosciences, Breda, the Netherlands), and erythrocytes (anti–TER-119 antibody; BD Pharmingen, Breda, the Netherlands). The signal was visualized by immunofluorescence-labeled secondary antibody and recorded with a Zeiss LSM510.NLO inverted laser scanning confocal microscope (Carl Zeiss, Thornwood, NY). Lipid deposition was analyzed using Oil Red O Staining (Sigma-Aldrich) and visualized by bright-field microscopy.

**MC Culture Conditions**

MC9 cells (Lanza, Breda, the Netherlands; catalog No. CRL8306) were grown in modified DMEM (supplemented with 4.5-g/L glucose, 544-mg/L L-glutamine, 1.5-g/L NaBic, 5-mmol/L mercaptoethanol; Lanza) and supplemented with 10\% Rat T-stim (BD Biosciences), 10\% fetal calf serum, and 1\% penicillin/streptomycin. Cells were plated in a 24-well plate (1×10\^6 cells/mL per well). Cells were activated with 100-ng E. coli LPS with or without preincubation with either \textit{B. quintana} LPS or an anti-TLR4a. Cells were preincubated for 30 minutes before adding E. coli LPS. We harvested medium and cells after 24 hours of stimulation, centrifuged medium and cells, collected supernatant, and stored supernatant until further use. Every experiment was conducted 3× with triplicate samples.

**IL-6 and IL-1β ELISA**

Murine IL-6 and IL-1β levels were measured by commercially available ELISAs (BD Biosciences). Ninety-six-well plates were coated with anti-mouse IL-6 capture antibody and incubated overnight at 4°C. The wells were washed 3× (PBS with 0.05\% TWEEN 20) and blocked for 1 hour at room temperature with 200-μL assay diluent (PBS with 10\% fetal bovine serum). Wells were washed 3×, and 100-μL standard or sample was added to each well and incubated for 2 hours. Wells were washed 5×, and 100-μL biotinylated anti-mouse IL-6 was added and incubated for 1 hour. Wells were washed 7×, and 100-μL streptavidin horseradish peroxidase conjugate was added and incubated for 30 minutes. A stop solution of 50 μL was added to each well (1M-H 3PO4), and colored product was read at 450 nm within 30 minutes with a correction 570 nm. IL-1β was measured with a similar protocol.

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DNP-HSA indicates 2,4-dinitrophenyl-human serum albumin; TLR4a, toll-like receptor 4 antibody; \textit{B. quintana} LPS, \textit{Bartonella quintana} lipopolysaccharide.
**VSMC Coculture With MC/9 Cells**

Murine VSMCs were isolated from the aorta and grown in DMEM (Lonza; catalog No. CRL8306) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. MC/9 cells were grown as described previously. After 1 week, VSMCs (1×10^6 cells/mL per well) were plated in a transwell 12-well plate with insert (Costar, Landsmeer, the Netherlands). After 24 hours, the inserts were filled with, respectively, 1-mL medium with or without 100-ng *E. coli* LPS (serotype O55:B5; Sigma-Aldrich) or IL-6, MC/9 cells (1×10^6 cells/mL, 1 mL), MC/9 cells with 100-ng *E. coli* LPS, MC/9 cells with 100-ng *E. coli* LPS and preincubation with TLR4a (10 µg/mL), MC/9 cells with 100-ng *E. coli* LPS and preincubation with B. quintana LPS (10 µg/mL), MC/9 cells treated with murine MC protease 4 small interfering RNA and with 100-ng *E. coli* LPS, and MC/9 cells treated with murine IL-6 small interfering RNA and with 100-ng *E. coli* LPS. After 48 hours of coculture, VSMCs were harvested and analyzed for apoptosis using a commercially available Annexin V staining kit (BD Biosciences). Every experiment was conducted 3× with triplicate samples.

**Annexin V Staining of Murine VSMCs**

The insert of the transwell dish was discarded, and the supernatant of the VSMCs was collected and transferred to 15-mL tubes. Cells were washed with PBS and incubated with trypsin/EDTA (Lonza) for 5 minutes. Medium was added, and medium and cells were transferred to the corresponding 15-mL tubes. We centrifuged the tubes at 400g for 5 minutes, discarded the supernatant, and incubated the cells with 100-µL fluorescence-activated cell sorter buffer, 5-µL propidium iodide, and 5-µL fluorescein isothiocyanate. Annexin V was added to the cells for 15 minutes at room temperature. A 400-µL binding buffer 1× was added, and 10,000 cells were analyzed by flow cytometry (FACScanto, BD Biosciences). Cells that stained positive for fluorescein isothiocyanate Annexin V and propidium iodide are identified as late apoptotic cells and were used for analysis. Data were analyzed using FlowJo (Tree Star Inc, Ashland, OR).

**Quantification and Statistical Analysis**

Data analysis was performed using an automated commercial image analysis system (Impak C; Clemex Technologies, Longueuil, Quebec, Canada) to evaluate differences in plaque phenotype upstream of the cast among the different treatment groups. Hematoxylin–eosin staining was used to measure intima/media ratio and evaluate cap thickness and necrotic core size, α-actin staining to evaluate VSMC content, TUNEL staining to measure apoptotic cell percentage, CD31 staining to evaluate neovascularization, CD68 staining to evaluate macrophage content, and TER119 staining to assess intraplaque hemorrhaging. To compare apoptotic cell percentage in the cap and core region, we made an overlay of anti–α-actin staining and corresponding TUNEL staining, TUNEL-positive cells in the α-actin-positive area were regarded as apoptotic cells in the cap region, whereas TUNEL-positive cells outside the actin-positive region were considered as apoptotic cells in the core region. We tested with 1-way ANOVA and a subsequent Student Newman-Keuls multiple comparisons test. Two-tailed Student *t* tests were used to compare individual groups. Data are presented as mean±SEM. *P*<0.05 were considered significantly different.

**Results**

**TLR4 Inhibition Reduces MC Activation But Does Not Affect the Absolute Number of Recruited MCs In Vivo**

To examine the effect of local DNP-HSA treatment on MC recruitment and activation, we examined crossections with toluidine blue staining to detect resting and activated adventitial MCs. As expected, DNP-HSA treatment significantly increased the absolute number of adventitial MCs by 115% (Figure 1A and 1E) and the percentage of activated MCs by 88% (Figure 1F). Cotreatment of DNP-HSA with the natural TLR4 antagonist *B. quintana* LPS did not influence the absolute number of adventitial MCs (Figure 1B and 1E) but significantly reduced the percentage of activated MCs by 33% (Figure 1B and 1F). Monotherapy with *B. quintana* LPS or an anti-TLR4a did not change MC recruitment and activation in the plaque (Figure 1E and 1F). These data suggest that MC activation in our murine vulnerable plaque model is regulated via TLR4 signaling.

**MC Recruitment and Activation Does Not Influence Atherosclerotic Lesion Growth But Promotes Atherosclerotic Plaque Destabilization**

To assess the effect of MC recruitment and activation on atherosclerotic lesion development, we evaluated the intima/media ratio. There was no difference in intima/media ratio among control, DNP-HSA, or combined DNP-HSA and *B. quintana* LPS-treated mice (Figure 1C, 1D, and 1G). Moreover, monotherapy with *B. quintana* LPS or TLR4a did not influence the intima/media ratio (Figure 1G). Taken together, these data indicate that MC recruitment and activation by DNP-HSA does not affect lesion size.

The effect of MC recruitment and activation on plaque phenotype was further analyzed by immunohistochemistry. DNP-HSA treatment significantly reduced VSMC content by 87%. Remarkably, cotreatment with *B. quintana* LPS prevented MC-associated loss in VSMC content (Figure 2A). As shown in Figure 2A, VSMCs were mainly lost in the cap region, resulting in weakening of the fibrous cap. Apoptotic cell percentage in atherosclerotic plaques in DNP-HSA–treated mice was significantly increased by 63%, as identified by TUNEL assay and mainly located in the cap region (Figure 2B).

Cotreatment with *B. quintana* LPS again counteracted this increase in apoptotic cell percentage. Loss of VSMCs was also reflected in cap thickness and necrotic core size because MC activation reduced cap thickness by 25% and increased necrotic core size by 120%, whereas TLR4 inhibition by *B. quintana* LPS normalized cap thickness and necrotic core size (Figure 1H and 1I). Further analysis of plaque phenotype showed that MC recruitment and activation did not alter neovascularization, macrophage content, or lipid deposition (Figure 2C and 2E). There was also no difference in intraplaque hemorrhaging detected (data not shown). These data indicate that MC recruitment and activation through TLR4 activation mediates VSMC apoptosis in the fibrous cap overlying the necrotic core.

**MCs Promote Plaque Destabilization In Vivo in a TLR4-Dependent Manner**

We further validated the involvement of TLR4 and MCs in plaque destabilization with 2 additional experiments. In the first experiment, we validated the involvement of MCs in the TLR4-mediated plaque destabilizing effect. The MC stabilizer cromolyn was used to test DNP-HSA–induced MC recruitment and VSMC apoptosis. Cromolyn treatment significantly decreased the total number of perivascular adventitial MCs by 250% compared with PBS treatment (Figure 3A) but not the percentage of activated MCs (Figure 3B). This
reduction in adventitial MCs by cromolyn promoted VSMC numbers in the plaque (Figure 3C, 3E, and 3F). Furthermore, there was a reduction in the number of apoptotic cells in the cap region compared with PBS-treated animals, and a significant reduction in the core-residing apoptotic cells was also observed (Figure 3D). These data clearly show that the effect of TLR4 on plaque destabilization was indeed mediated by activated MCs.

Figure 1. Representative sections stained for toluidine blue (top) and hematoxylin-eosin (bottom) of a 2,4-dinitrophenyl-human serum albumin (DNP-HSA; A and C) and Bartonella quintana lipopolysaccharide (LPS)/DNP-HSA-treated lesion (B and D). Arrows indicate activated, degranulated mast cells whereas circles indicate resting, nondegranulated mast cells. E, Quantification of adventitial mast cells. DNP-HSA treatment increased the absolute mast cell numbers \((P<0.01)\). F, Quantification of the percentage activated mast cells. Local DNP-HSA increased the percentage of activated mast cells \((P<0.01)\), which was inhibited by \(B.\ quintana\) LPS \((P<0.01)\). G, The intima/media ratio was not affected. H, Relative necrotic core size was increased on DNP-HSA, which could be prevented by cotreatment with \(B.\ quintana\) LPS. I, Reversely, DNP-HSA decreased relative cap thickness, and this again could be prevented by cotreatment with \(B.\ quintana\) LPS. Data are presented as mean±SEM \((n=8\) per group). BartLPS indicates \(B.\ quintana\) LPS; TLR4, toll-like receptor 4.
Figure 2. Effect of mast cell activation on plaque phenotype. A, Adventitial recruitment and activation of mast cells reduced vascular smooth muscle cell (VSMC) percentage in the plaque ($P<0.05$), which was prevented by cotreatment with the toll-like receptor 4 (TLR4) antagonist *Bartonella quintana* lipopolysaccharide (LPS; $P<0.05$). B, Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay showed increased apoptotic cell percentage in 2,4-dinitrophenyl-human serum albumin (DNP-HSA)–treated mice ($P<0.05$), which was prevented by cotreatment with *B. quintana* LPS ($P<0.05$). C, DNP-HSA treatment was not associated with a change in (C) neovascularization, (D) percentage macrophages, or (E) lipid deposition. *Denotes the lumen. Data are presented as mean±SEM (n=8 per group). BartLPS indicates *B. quintana* LPS.
In a second control experiment, we used short hairpin RNA to target TLR4 and to induce gene silencing in MCs to validate the importance of TLR4 activation. Lentiviral transfection of the TLR4 short hairpin RNA combined with DNP-HSA did not influence the number of periadventitial MCs (Figure 4A) but led to a significant decrease in activated periadventitial MCs (Figure 4B) compared with sham-treated controls, which coincided with a decline in the number of apoptotic VSMCs in the cap region. A nonsignificant decrease of apoptotic cells in the core was also observed (Figure 4C–4F). Based on these findings, we conclude that MC-mediated cap thinning in the advanced atherosclerotic lesion is dependent on TLR4 activation.

MCs Induce VSMC Apoptosis via TLR4 Activation and Subsequent Chymase Release In Vitro

To further assess the role of MCs in VSMC apoptosis, we cocultured murine VSMCs with murine MCs (MC/9 cells) in a 0.4-µm transwell culture dish. MCs were activated with 100-ng E. coli LPS, a TLR4 agonist, with or without the natural TLR4 antagonist B. quintana LPS or a TLR4a. MCs activated by E. coli LPS increased VMSC apoptosis by 53% (Figure 5A, 5B, and 5E), whereas E. coli LPS did not have a direct apoptotic effect on a monoculture of VSMCs (Figure 5D and 5E). Induction of VSMC apoptosis by activated MCs was inhibited by preincubation with TLR4a, as well as B. quintana LPS (Figure 5C and 5E).

As Bot et al27 recently pointed out that a potential role of chymase in determining plaque stability and as Leskinen et al10 and Guo et al18 showed that chymase may induce VSMC apoptosis in vitro and in vivo, respectively, we cocultured E. coli LPS-activated MCs with VSMCs in the presence or absence of a chymase inhibitor (soy bean trypsin inhibitor, 100 µg/mL). Soy bean trypsin inhibitor inhibited VSMC apoptosis induced by TLR4-activated MCs (Figure 6A), indicating a role for TLR4 signaling in chymase-induced VSMC apoptosis. The role of chymase in the TLR4 activation pathway in MCs was further validated by the use of small interfering RNA-mediated silencing of MC protease 4, the murine equivalent of human chymase. VSMC apoptosis by E. coli LPS-activated MCs was prevented by pretreatment of MCs with small interfering MC protease 4 (Figure 5E), indicating that chymase plays an important role in this process.
Kirshenbaum et al.22 showed that *E. coli* LPS can increase chymase expression in mature human MCs via IL-1β and IL-6 autocrine stimulation. We, therefore, hypothesized that TLR4 signaling is involved in chymase-induced VSMC apoptosis via IL-1β and IL-6 induction and examined cytokine production by MCs activated with *E. coli* LPS. Stimulation of MCs with 100-ng *E. coli* LPS significantly increased IL-6 cytokine production from not being detectable in control conditions to 393.5±46.6 pg/mL (Figure 6A), which was abolished by preincubation with 10-µg *B. quintana* LPS or 10-µg TLR4a (Figure 6B). However, MCs failed to produce IL-1β on stimulation with 100-ng *E. coli* LPS (data not shown). To further validate the importance of IL-6 in the process of MC-induced VSMC apoptosis, MC/9 cells were pretreated with small interfering IL-6. Silencing of IL-6 significantly diminished VSMC apoptosis induced by *E. coli*-activated MC/9 cells (Figure 5E).

Next, we tested whether IL-6 induced VSMC apoptosis by either directly affecting VSMC survival or by autostimulation and activation of MCs. Monoculture of VMSC with IL-6 stimulation did not affect VSMC death whereas a similar dose of IL-6 (1000 pg) administrated to MCs in cocultures significantly increased VSMC apoptosis (Figure 6C).

We further assessed whether this VSMC apoptosis was attributable to the stimulation of chymase release by IL-6. Chymase inhibition by soy bean trypsin inhibitor was able to block MC-mediated VSMC apoptosis in response to IL-6 (Figure 6C). Thus, combined, these data clearly indicate that MC activation by TLR4 induced VSMC apoptosis by IL-6–regulated chymase production.

**Discussion**

Our study shows for the first time that TLR4 activation on MCs is involved in MC-induced atherosclerotic plaque destabilization by VSMC apoptosis in vivo and in vitro. This apoptosis is dependent on MC IL-6 production and subsequent chymase release in an autocrine fashion. To test our hypothesis, we combined a well-established model for MC recruitment and activation in murine atherosclerosis with our vulnerable plaque model in apolipoprotein E knockout mice.5,24 Local recruitment and activation of MCs specifically induced VSMC apoptosis without affecting other vulnerable plaque parameters, such as lipid and macrophage content or neovascularization. Inhibition of TLR4 signaling, using the natural TLR4 antagonist *B. quintana* LPS,25 reduced the number of activated MCs and
was able to prevent MC-induced VSMC apoptosis. These results were subsequently validated in 2 control experiments using short hairpin RNA-mediated silencing of TLR4 and the MC stabilizer cromolyn. This suggests a role for TLR4 in MC-induced VSMC apoptosis and cap thinning–related destabilization of vulnerable plaque. The lack of effect on the percentage of VSMCs in the vulnerable lesion in the *B. quintana* LPS and TLR4a monotherapy-treated groups...
points toward a specific role for TLR4 in MC-related vulnerable plaque destabilization. In addition, TLR4 inhibition did not alter intimal accumulation of other cell types, including macrophages and endothelial cells. MCs have been suggested to be involved in the initiation and progression of atherosclerotic disease and could potentially influence plaque phenotype by producing a broad variety of bioactive components, including cytokines (eg, IL-6 and tumor necrosis factor), 28 chymase, tryptase and histamine, and activating matrix metalloproteinases. 12,17 In relation to vulnerable plaque, it has been shown that MCs accumulate predominantly in the shoulder region of the advanced atherosclerotic lesions, the region most prone to rupture. 14 However, studies that describe the role of MCs in atherosclerosis in vivo and clearly demonstrate a causative role for this type of cells in vulnerable plaque are limited. It was already shown that MCs could induce VSMC apoptosis 10 in vitro and that MC chymase is responsible for this phenomenon. Since this first observation, different groups have studied the pathway downstream of chymase, leading to VMSC apoptosis. Leskinen et al 15 showed that chymase induces degradation of fibronectin, which causes disruption of focal adhesion interaction with the extracellular matrix, leading to loss of focal adhesion kinase activation and inhibition of the outside-in signaling, which ultimately leads to apoptosis of cells. The exact mechanism of MC activation and subsequent release of chymase in atherosclerosis is not yet known. Kirshenbaum et al 22 showed, in vitro, that E. coli LPS upregulates chymase expression on human MCs via IL-1β and IL-6 production because those cytokines by themselves or in combination were also able to upregulate chymase expression. Our in vitro data clearly indicate that TLR4 activation on MCs is involved in chymase release and thus VSMC apoptosis.

**Figure 6.** Mast cell–induced vascular smooth muscle cells (VSMCs) apoptosis via toll-like receptor 4 (TLR4) signaling. A, Preincubation with chymase inhibitor, soy bean trypsin inhibitor (SBTI) normalized mast cell–mediated VSMC apoptosis (P<0.001). B, Stimulation of MC/9 cells with 100-ng Escherichia coli lipopolysaccharide (LPS) induced interleukin-6 (IL-6) production (P<0.001), which was inhibited by preincubation with Bartonella quintana LPS (P<0.001) and TLR4 antibody (TLR4a; P<0.05). C, MC/9 cells stimulated with IL-6 significantly increased VSMC apoptosis (P<0.01), whereas preincubation with SBTI inhibited this effect (P<0.05). Data are presented as mean±SEM. Data represent 3 different experiments. D, Suggested mechanism of TLR4 activation on mast cells leading to VSMC apoptosis in atherosclerosis. Activation of TLR4 on mast cells leads to nuclear translocation of nuclear factor κB (NF-κB) and consequently transcription of proinflammatory cytokines, including IL-6. IL-6 triggers chymase release via autocrine and paracrine stimulation and subsequent VSMC apoptosis. BartLPS indicates B. quintana LPS.
investigated whether we could replicate our in vivo data by coculturing MC/9 cells with VSMCs in the presence of E. coli LPS with or without preincubation with B. quintana LPS or TLR4a. We cocultured VSMCs and MC/9 cells in a transwell 12-well plate with 0.4-μm pore size inserts. In this setup, there was no mixing of VSMCs and MC/9 cells because the MC/9 cells seeded in the upper insert chamber remained separated from the VSMCs in the bottom chamber. However, bioactive compounds (eg, chymase) released by MCs on stimulation can diffuse to the lower chamber and exert their biological activity on the VSMCs. Conforming to the findings in our vulnerable plaque model, coculture of E. coli LPS-activated MC/9 cells with VSMCs increased VSMC apoptosis, and this could indeed be prevented by preincubation with 2 different TLR4 inhibitors, B. quintana LPS and TLR4a. This indicated that MCs induced apoptosis of VSMCs by the release of a paracrine factor. Preincubation of MCs with the chymase inhibitor, soy bean trypsin inhibitor reduced VSMC apoptosis significantly, suggesting that indeed chymase is involved in MC-induced VSMC apoptosis. Likewise, pretreatment of MC/9 cells with small interfering MC protease 4 also prevented VSMC apoptosis.

Based on the study of Kirshenbaum et al22 and the fact that TLR4 activation leads to nuclear translocation of nuclear factor κB and subsequently transcription of proinflammatory cytokines, including IL-620 we suggested that IL-6 can autostimulate the MC to release chymase. TLR4 activation on MC/9 MCs indeed led to IL-6 production, and this was blocked by B. quintana LPS and to a lesser extent by TLR4a. Furthermore, knockdown of IL-6 in MC/9 cells led to less VSMC apoptosis. We could also demonstrate that TLR4 activation on MCs could lead to VSMC apoptosis via IL-6–induced chymase release. Together, these data clearly indicate that MC-induced apoptosis of VSMCs is controlled by TLR4 signaling via autocrine IL-6/chymase release mechanism and points toward a plaque destabilizing role of MCs by chymase release. In line with our findings, Bot et al have recently demonstrated that chymase inhibition by RO5066852 could indeed help to stabilize advanced atherosclerotic lesions. Our findings further strengthen the notion that MCs play an important role in determining plaque stability via different pathways.

A limitation of the study is that we used an artificial model to create a vulnerable plaque in the mouse. We are aware that the brachiocephalic artery can be used as a natural model to study the vulnerable plaque. However, this site is not accessible for perivascular intervention.

In conclusion, we demonstrate that TLR4 signaling is important for MC-induced VSMC apoptosis. TLR4 activation leads to IL-6 production, which in turn autostimulates the MC to release chymase. Finally, chymase induces VSMC apoptosis, thus destabilizing the atherosclerotic plaque by thinning of the fibrous cap (Figure 6D).

Disclosures

None.

References


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